

Amendments to the Specification:

The following is a marked-up version the Specification pursuant to revised 37 C.F.R. §1.121, with instructions and markings showing changes made herein to the Specification as filed. Underlining denotes added text while brackets denote deleted text.

At page 1, after the Title, please add the following paragraphs:

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant No. 70NANB5H1138 awarded by Advanced Technology Program/National Institute of Standards and Technology. The Government has certain rights in this invention.

This is a Divisional of co-pending U.S. Patent Application Serial No. 09/470,830, filed on December 23, 1999.

Please replace the "SUMMARY OF THE INVENTION" located at pages 2 - 4 of the Specification with the following:

SUMMARY OF THE INVENTION

The present invention generally relates to chaperonin protein binding domains and to the use of an isolated chaperonin protein binding domain in the production of heterologous proteins, peptides or polypeptides in a host cell. The present invention is based, in part, upon the finding that a toxic gene product could be recombinantly produced by a host cell when expressed as a fusion protein associated with an isolated chaperonin binding domain.

Accordingly, the present invention provides a method for producing a protein in a host cell, comprising the step of culturing a host cell comprising a first nucleic acid encoding an isolated chaperonin binding domain associated with a second nucleic acid encoding the protein and a third nucleic acid encoding a chaperonin under conditions suitable for expression of said first, said second and said third nucleic acid and wherein

said chaperonin binding domain is capable of binding to said chaperonin. In a further embodiment, the chaperonin binding domain and the chaperonin are capable of binding with an affinity of between about 10^{-2} and 10^{-8} Kd. The method may further comprise recovering said protein from said cell. In one aspect, the protein is one toxic to the host cell. A protein may be toxic to a host cell due to its intrinsic nature or toxic due to the presence of elevated levels in the host cell.

In another embodiment of the present invention, the first and second nucleic acid encode a fusion protein. The first and second nucleic acid may be directly linked or indirectly linked by nucleic acid encoding an enzymatic cleavage site, a chemical cleavage site, or another protein or peptide.

In one aspect of the invention, nucleic acid encoding the chaperonin is naturally produced by the host cell and the cell is grown under conditions that result in elevated levels of the chaperonin. In another aspect, nucleic acid encoding the chaperonin is heterologous to the host cell and the heterologous chaperonin is under the control of at least one expression signal capable of overexpressing the chaperonin in the host cell. The present invention encompasses any host cell that is capable of expression of recombinant proteins. In one embodiment, the host cell is a bacterium. In another embodiment, the host cell is a eubacterium. In yet further embodiments, the host cell is a gram-positive or a gram-negative bacterium. In a further embodiment, the bacterial cell is a member of the family *Enterobacteriaceae*. In an additional embodiment, the bacterial cell is an *Escherichia* species, in particular *E. coli*.

There are several well characterized chaperonin systems known in the art having two or more interacting partners, for example, Hsp60 and Hsp10 (GroEL/GroES); Hsp70 and Hsp40 and GrpE (DnaK/DNAJ/GrpE); ClipA/X and ClipP; Hsp90 and Hsp70 and other factors; TriC and other factors. The present invention encompasses chaperonin binding domains obtainable from these systems as long as the chaperonin binding domain is capable of binding to a chaperonin with an affinity of between about 10^{-2} and 10^{-8} Kd. In one embodiment, the chaperonin binding domain has the sequence as shown in [SEQ ID NO: 1 through SEQ ID NO: 38] SEQ ID NO: 3 through SEQ ID NO: 40. In yet another embodiment, the chaperonin binding domain is obtainable from the GroES co-chaperonin and said chaperonin is the GroEL chaperonin. In another embodiment, the binding domain comprises the amino acid sequence EVETKSAGGIVLTGSAAA, (SEQ ID NO: 2). In a further embodiment, the binding domain comprises a variation of the sequence EVETKSAGGIVLTGSAAA, (SEQ ID NO:

2) said variant being capable of binding to GroEL chaperonin with an affinity of 10^{-2} to 10^{-8} Kd. The present invention also provides expression vectors and host cells comprising a chaperonin protein binding domain.

Examples of heterologous proteins include therapeutically significant proteins, such as growth factors, cytokines, ligands, receptors and inhibitors, as well as vaccines and antibodies; enzymes such as hydrolases including proteases, carbohydrases, and lipases; isomerases such as racemases, epimerases, tautomerases, or mutases; transferases, kinases and phosphatases; and commercially important industrial proteins or polypeptides, such as proteases, carbohydrases such as amylases and glucoamylases, cellulases, oxidases and lipases. The nucleic acid encoding the heterologous protein may be naturally occurring, a variation of a naturally occurring protein or synthetic.

Please replace the entire paragraph defining "isolated binding domain" in the definition section under DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS at page 5, line 32 through page 6, line 14 of the Specification with the following:

As used herein, the phrase "isolated binding domain" of a chaperonin or "chaperonin protein binding domain" or "chaperonin binding domain" refers to a region of a protein or polypeptide that is able to bind with an affinity of between 10^{-2} and 10^{-8} Kd to a chaperonin or portion or fragment thereof of said chaperonin. In one embodiment of the present invention, the chaperonin binding domain has the sequence as shown in any of [SEQ ID NO: 1 through SEQ ID NO:38] SEQ ID NO: 3 through SEQ ID NO: 40. In another embodiment of the present invention, the chaperonin binding domain is obtainable from the GroES co-chaperonin. As used herein, the chaperonin protein binding domain obtainable from GroES refers to the residues shown in Figure 1 comprising the sequence EVETKSAGGIVLTGSAAA, SEQ ID NO: 2. In another embodiment, the binding domain comprises amino acid variations of EVETKSAGGIVLTGSAAA, SEQ ID NO: 2 capable of binding to a GroEL chaperonin with an affinity of between 10^{-2} and 10^{-8} Kd. A chaperonin protein binding domain is associated with a second nucleic acid encoding a heterologous protein when the first and second nucleic acids are directed linked, such as in a fusion protein, or are

indirectly linked such as having an enzymatic cleavage site, chemical cleavage site or other nucleic acid inserted between the first and the second nucleic acid.

Please replace Section II. Chaperonin binding domain including Table 1 located at pages 9 and 10 of the Specification with the following:

II. Chaperonin binding domain

The present invention encompasses chaperonin binding domains that are capable of binding to a chaperonin with an affinity of between 10^{-2} and 10^{-8} Kd. Examples of chaperonin binding domains are provided in Table I. Table I provides the sequence of chaperonin binding domains and a list of the respective microorganism from which the binding domain is obtained (Hunt et al., 1996, Nature vol. 379, pages 37-45).

Table 1.

Organism	Chaperonin binding domain	Sequence Identifier (SEQ ID NO:
ch10_ecoli	EVETKSAGGIVLTGSAAAK	SEQ ID NO: 3
ch10_acype	EVESKSAGGIVLTGSAAGK	SEQ ID NO: 4
ch10_haedu	EVETCSAGGIVLTGSATVK	SEQ ID NO: 5
ch10_pseae	EEETKTAGGIVLPGSAAEK	SEQ ID NO: 6
ch10_chrvi	EEERLSAGGIVIPDSATEK	SEQ ID NO: 7
cg10_coxbu	EEERTSAGGIVIPDSAAEK	SEQ ID NO: 8
ch10_legmi	EEERTTAGGIVIPDSATEK	SEQ ID NO: 9
ch13_rhime	ESEEKTKGGIIIPDTAKEK	SEQ ID NO: 10
ch10_legpn	EEERTTAGGIVIPDSATEK	SEQ ID NO: 11
ch10_bruab	ESEAKTAGGIIIPDTAKEK	SEQ ID NO: 12
ch12_braja	DAEEKTAGGIIIPDTVKEK	SEQ ID NO: 13
ch10_agrtu	ESEAKTKGGIIIPDTAKEK	SEQ ID NO: 14
ch10_cloab	EAEETTKSGIVLPSSAKEK	SEQ ID NO: 15
ch10_amops	EEERTTAGWIVIPDSATEK	SEQ ID NO: 16
ch11_rhime	ESEEKTKGGIIIPDTAKEK	SEQ ID NO: 17
ch10_lacla	EEEEKSMGGIVLTSASQEK	SEQ ID NO: 18
ch10_stral	DAEQTTASGLVIPDTAKEK	SEQ ID NO: 19
ch10_thep3	ETEEKTASGIVLPDTAKEK	SEQ ID NO: 20
ch10_bacsu	ESEEKTASGIVLPDSAKEK	SEQ ID NO: 21
ch10_bacst	ETEEKTASGIVLPDTAKEK	SEQ ID NO: 22
ch10_myctu	EAETTTASGLVIPDTAKEK	SEQ ID NO: 23
ch13_braja	DAEEKTAGGIIIPDTAKEK	SEQ ID NO: 24

ch10_staau	EQEQTTSKSGIVLTDSAKEY	SEQ ID NO: 25
ch10_mycbo	EAETTTASGLVIPDTAKEK	SEQ ID NO: 26
ch10_mytle	EAETMTPSGLVIPENAKEK	SEQ ID NO: 27
ch10_clope	EAEETTKSGIIVTGTAKER	SEQ ID NO: 28
ch10_synp7	EAEKTAGGIILPDNAKEK	SEQ ID NO: 29
ch10_synp6	EAEKTAGGIILPDNAKEK	SEQ ID NO: 30
ch10_syny3	PAEEKTAGGILLPDNAKEK	SEQ ID NO: 31
ch10_chlpn	EEEATARGGIILPD TAKKK	SEQ ID NO: 32
ch10_lepin	QEAEKIGSIFVPDTAKEK	SEQ ID NO: 33
ch10_chlps	EEDSTARGGIILPD TAKKK	SEQ ID NO: 34
ch10_chltr	EEASTARGGIILPD TAKKK	SEQ ID NO: 35
ch10_rat	AAETVTKGGIMLPEKSQ GK	SEQ ID NO: 36
ch10_bovin	AAETVTKGGIMLPEKSQ GK	SEQ ID NO: 37
ch10_ricts	QNDE. AHGKILIPDTAKEK	SEQ ID NO: 38
ch10_spiol	EVENKTSGGLLLAESSKEK	SEQ ID NO: 39
ch10_arath	IQPAKTESGILLP . EKSSK	SEQ ID NO: 40

In a preferred embodiment, the chaperonin binding domain is the sequence EVETKSAGGIVLTGSAAA (SEQ ID NO: 2) or portions or variations thereof which bind to the GroEL chaperonin with an affinity of between about 10^{-2} to about 10^{-8} Kd.

For construction of a fusion protein, the chaperonin binding domain may be directly linked to the desired protein, peptide or polypeptide, or indirectly linked, ie comprising additional nucleic acid between the nucleic acid encoding the chaperonin binding domain and the protein or peptide or polypeptide. Such additional nucleic acid may encode enzymatic cleavage sites or chemical cleavage sites. Nucleic acid encoding the chaperonin may be 5' or 3' to the nucleic acid encoding the protein, peptide or polypeptide.

Please replace the section "Construction of vectors for expressing chaperonin binding domain fusion proteins" in the Materials and Methods section at pages 13 - 14 of the Specification with the following:

Construction of vectors for expressing chaperonin binding domain fusion proteins

Vectors designed to comprise the chaperonin binding domain obtainable from GroES were constructed as follows. Oligonucleotides were synthesized based on the published amino acid sequence of the *E. coli* GroES protein. Residues 16 through 33

comprise the chaperonin binding domain EVETKSAGGIVLTGSAAA, SEQ ID NO: 2. A nucleotide sequence encoding this sequence and its complement were generated by the program Lasergene (DNASTar, Inc., Madison, WI.) using the general codon preferences for *E. coli*. A linker was designed to require the same reading frame as that required in pASK40. Additional nucleotides encoding the overhang generated by an EcoRI digest of DNA and an ATG initiation codon were included at the 5'-end of each to give:

Oligo ATP6: 5'-AATTATGGAAGTTGAAACCAAATGTGCTGGTGGTATCG-
-TTCTGACCGGTTCTGCTGCTGCG-3', SEQ ID NO: 44
Oligo ATP7: 5'-AATTCGCAGCAGCAGAACCGGTCAGAACGATACCACCA-
-GCAGATTTGGTTTCAACTTCCAT-3', SEQ ID NO: 42

The design of the linker for attachment of the GroEL-binding domain of GroES to proteins is shown in Figure 5 (SEQ ID NO: 43).

The linker was phosphorylated using T4 polynucleotide kinase, purified with Qiaex resin (Qiagen), and ligated into the dephosphorylated EcoRI site of the vector pJF118EH. Transductants of strain JM105 were screened for the presence of an EcoRI site, which is present in the linker but absent in pJF118EH. Positive transductants were screened for orientation of the insert. Only one end regenerates an EcoRI site, and in the correct orientation that site is adjacent to a multicloning site. EcoRI-EcoRV digests were analyzed on a 1.5% agarose gel for the presence of the 1,260 base pair fragment predicted for the correct orientation as opposed to the 1,200 base pair fragment predicted for the reverse orientation. The new vector was designated pATP004.

Because the multi-cloning site of pATP004 was limited in the number of sites available for cloning, we exchanged this cluster for the larger cluster of pUC19 using the enzymes EcoRI and HindIII. Plasmids obtained from JM105 transductants were screened for orientation, as above, and for the presence of the KpnI and XbaI sites present only in the pUC19 multicloning site. The resulting plasmid was designated pATP005.

Please replace Example 1 at pages 15 - 16 of the Specification with the following:

Construction of Expression Vector

A synthetic linker (SEQ ID NO: 41) designed to encode the chaperonin binding loop of GroES (EVETKSAGGIVLTGSAAA) (SEQ ID NO: 2) was ligated into the EcoRI site of plasmid pJF118EH. Plasmid DNA was prepared from representative colonies arising from transformation of *E. coli* JM105 with this ligation mixture, and screened for the presence of an AgeI site, which is unique to the introduced linker. Of 10 colonies screened, all contained the site. The plasmids were then screened for orientation of the linker, since it could be incorporated in two directions. The linker was designed so that only one EcoRI site would be regenerated, which in the desired orientation would be attached to the multi-cloning site. Plasmids were digested with EcoRI and EcoRV (present in the *tac* promoter) and analyzed on a 1.5% gel. The desired orientation, present in 5 of the plasmids, generated a 1,260 base-pair fragment; those in the wrong orientation, with the reconstituted EcoRI site at the downstream side of the inserted linker, generated a fragment of 1,200 basepairs. A representative of the correct orientation was propagated and designated pATP004.

To expand the potential of the vector, the small multi-cloning site of pAF118EH was excised from pATP004 by digesting it with EcoRI and HindIII, and replaced with the EcoRI-HindIII multi-cloning site of pUC19. Of eight colonies screened, seven contained the inserted multi-cloning site. These were further shown to have the expected orientation and the additional restriction sites. A representative colony was designated pATP005.